Remarks

Claims 1-39 were the subject of the office action dated July 7, 2007. Claims 10, 13-14, 24, 28-29, and 34 are canceled without prejudice in favor of the remaining claims and to simplify the issues before the examiner. For example, claims 13 and 14 are canceled in favor of claim 1 as amended. Claims 40-42 are added. Thus, claims 1-9, 11-12, 15-23, 25-27, 30-33, and 35-42 are now before the examiner for further consideration.

Although the applicant believes that the examiner has cited art that summarizes the state of the art when the subject invention was made, as discussed in more detail below, an Information Disclosure Statement will be submitted under separate cover.

Replacement Figures are submitted herewith.

Sequence identifiers are now provided as suggested by the examiner.

Claim 26 is amended to correct the dependency. Claim 32 is amended to correct the typographical error. No typographical error was observed in claim 33. The typographical error is corrected in claim 38.

Claims 23 is amended, which renders moot the indefiniteness rejection. Claim 24 is similarly canceled. Claims 32 and 38 are amended to render moot the indefiniteness rejections of those claims.

The applicant respectfully traverses the §102(b) rejections in light of WO 99/10513 and in light of Daniell et al (2001; Curr. Genet. 39:109-116).

The applicant, Dr. Henry Daniell, also the inventor of WO 99/10513 confirmed that WO 99/10513 does not teach somatic embryogenesis. While a short passage of this reference is cited, WO 99/10513 does not (inherently) enable somatic embryogenesis, contrary to the statements in the

last paragraph on page 5 of the office action. The same is true for the cited Daniell et al. literature reference. The detailed remarks provided below explain this further.

100% identical (or homologous), species-specific vectors are used according to the subject invention. This is now specified in claim 1, for example. In addition, a promoter region function in green and non-green tissues and in light and dark conditions is now specified in claims 2 and 3. Claims 28-29 and 34, to some non-green plant cells, are canceled, which should also help to render moot this rejection.

In light of the foregoing, the rejections for anticipation should be rendered moot. The withdrawal of these rejections is thus requested.

The applicant respectfully traverses the §103(a) rejections in light of WO 99/10513 and in light of WO 99/10513 taken with Maliga (USPN 5,877,402).

The applicant, Dr. Henry Daniell, also the inventor of WO 99/10513 confirmed that WO 99/10513 does not teach somatic embryogenesis. While a short passage of this reference is cited, WO 99/10513 does not (inherently) enable somatic embryogenesis, contrary to the statements in the last paragraph on page 5 of the office action. An expert declaration from Dr. Daniell can be produced to this effect. Claim changes are discussed above with respect to the §102(b) rejections, which should also help to render these obviousness rejections moot.

Regarding Maliga, Maliga attempted to transform rice, but they failed. They showed only heteroplasmy (few transformed chloroplasts). In contrast, the applicant achieved transformation of all chloroplast genomes (homoplasmy) in each cell. Heteroplasmic lines are useless because they will lose their genes once the selection pressure is removed. Maliga repeatedly acknowledges that their rice plant is heteroplasmic.

As explained in more detail below, heteroplasmy is the first step of chloroplast transformation. There are a number of subsequent challenging steps in somatic embryogenesis.

Maliga did not show (or claim) chloroplast transformation in rice via somatic embryogenesis. These authors state (in Nature Biotechnology 17: 910-915, on page 913, left column) that "in rice only a small fraction of chloroplasts expressed FLARE-S" and that "individual cells contained a

mixed population of wild type and transgenic chloroplasts." The authors further confirm that "the transplastomic rice plants generated in this study are heteroplasmic." Even after several years, the same author states in his publication on page 298 (Maliga, Annual Review of Plant Biology, 2004, 55:289-313) that "rice plants regenerated from the transformed culture were heteroplasmic."

Each plant cell contains up to 10,000 copies of chloroplast genomes. In order to achieve successful chloroplast transformation, all 10,000 copies should have integrated foreign genes (homoplasmy) and there should not be a mixture of transformed and untransformed chloroplast genomes (heteroplasmy). Homoplasmy has been so far achieved in crops that produce shoots directly from leaves bombarded with foreign genes. When plants produce shoots directly from leaves, this process is described as organogenesis. When heteroplasmic condition is observed, these shoots are cut into small pieces and regenerated again under stringent selection conditions to eliminate untransformed chloroplasts or wild type chloroplast genomes. After several rounds of selection, homoplasmic shoots are obtained.

However, several plant species do not regenerate via organogenesis but each embryogenic cell forms an embryo. This is called a somatic (vegetative) embryo to distinguish it from embryos that are usually formed after sexual reproduction. The somatic embryo then gives rise to the shoot and root. It is not possible to chop embryos to small pieces and regenerate shoots to achieve homoplasmy. Although chloroplast transformation was achieved via organogenesis in 1990, until the subject invention, chloroplast transformation via somatic embryogenesis has been elusive and unsuccessful in several laboratories around the world. Major challenges to achieve chloroplast transformation via somatic embryogenesis included the use of appropriate regulatory sequences and selectable markers that function in non-green and green chloroplasts, ability to regenerate chloroplast transgenic plants via somatic embryogenesis and achieve homoplasmy, which lacks the benefit of subsequent rounds of regeneration offered by organogenesis. Understanding and manipulating the somatic embryogenesis system, which lacks the advantage of subsequent rounds of regeneration from heteroplasmic tissues, was a major challenge.

During transformation, transformed non-green plastids should develop into mature chloroplasts and transformed cells should survive the selection process during all stages of development. Therefore, a major challenge was to provide plastids an ability to survive selection in

the light and the dark, at different developmental stages. This is important because only one or two chloroplasts are transformed in a plant cell after bombardment, and these plastids should have the ability to survive the selection pressure, and multiply and establish themselves while all other untransformed plastids are eliminated in the selection process. Therefore, chloroplast transformation involves creation of suitable chloroplast vectors, DNA delivery into competent recipient cells, integration into one or two chloroplast genomes (heteroplasmy), replacement of all native (wild type) chloroplast genomes with transformed chloroplast genomes to achieve homoplasmy, induction of somatic embryos from homoplasmic embryogenic cells and regeneration of transgenic plants. In the subject invention, the applicant developed for the FIRST time a complete process to achieve chloroplast transformation in plants that regenerate via somatic embryogenesis.

The applicant demonstrated this first in carrot and then in cotton, rice, and several other crops. First, species specific chloroplast transformation vectors were made. Carrot specific chloroplast transformation vector (pDD-*Dc-aad*A/*badh*) contains the *aad*A gene regulated by the 5' ribosome binding site (rbs) region or the Shine-Dalgarno sequence (GGAGG) / *psb*A 3'UTR and the *badh* gene regulated by the 5' ribosome binding site (rbs) region of the bacteriophage T7 gene 10 leader in order to facilitate expression in green as well as non-green tissues and the *rps*16 3'UTR to stabilize transcripts. Transcription of the expression cassette in carrot chloroplast transformation vector is driven by the full-length 16S rRNA promoter. The full-length promoter comprises of binding sites for both the plastid-encoded and nuclear-encoded RNA polymerase thereby facilitating transcription in green or non-green tissues.

Yellow (non-green) fine cell suspension culture induced from stem segments of carrot were bombarded with carrot chloroplast transformation vector pDD-*Dc-aad*A/*badh*. Chloroplast transformation protocol was optimized using different bombardment conditions to achieve reproducibility using different cell cultures. In order to optimize gene delivery, chloroplast transformation vectors were bombarded using rupture discs of different psi, at varying distances between rupture discs and the target tissues. Maximum transformation efficiency was achieved with carrot cell cultures bombarded at 1100 psi pressure and at a distance of 12 cm. Much lower efficiencies were obtained at other parameters used for particle bombardment.

Transgenic plants regenerated after two subcultures in selective liquid medium showed heteroplasmy as was evident by the presence of both 1.4 kb wild type and 3.2 kb transformed chloroplast genomes. Plants that were regenerated from cell lines after 8-10 subcultures in liquid medium supplemented with a high concentration of selection agent exhibited almost complete homoplasmy as only the 3.2 kb DNA fragment, representing only transformed chloroplast genomes. A very faint signal corresponding to the wild-type fragment was observed in cell lines that have not gone through repetitive stringent selection; subsequent rounds of selection eliminated this wild-type fragment.

Using the carrot chloroplast transformation vector, several independent transgenic cell lines were recovered using different sets of parameters for particle bombardment within 2-3 months from bombarded calli selected on solid medium containing lower levels of selection agent. Later, the transgenic calli were transferred to mid-range selection conditions for a month and subsequently multiplied under high stringent selection conditions. In order to further multiply the transgenic cell cultures, they were either subcultured on solid medium after every 2-3 week or rapidly multiplied in liquid medium maintained at 130 rpm under diffuse light (50 lux) after every week.

Using species-specific cotton chloroplast vectors, several independent resistant calli were obtained. All of these independent resistant calli were tested for stable transgene integration into the chloroplast genome by PCR or for homoplasmy by Southern blots. Transformed cell lines were also tested for their morphogenic response. Suitable cultures were chosen to produce elongated somatic embryos. Hypocotyl of elongated embryos was dissected into small pieces to induce callus and somatic embryos. Plants produced from transgenic lines were grown in the growth chamber. T1 seedlings from these lines were tested by Southern blots to evaluate homoplasmy. Transgenic lines maintained *in vitro* in the callus form, derived from hypocotyl of elongated somatic embryos were also observed to be homoplasmic. About 5-20 somatic embryos were derived from transgenic calli after each subculture. Therefore, generating several transgenic plants from a single culture is quite feasible.

In summary, transgenic calli were first selected on suitable medium containing low concentrations of selection agent after bombardment with chloroplast vectors. Selected cell cultures were repeatedly subcultured every month on higher selection medium, in order to increase the

number of transgenic chloroplasts in cell cultures (visually green in color). To induce the somatic embryogenesis, cell cultures were plated on suitable medium and well-differentiated somatic embryos were elongated on filter papers placed on suitable medium. Further, hypocotyls of elongated somatic embryos were dissected into small pieces and placed on selection medium to induce callus. Induced calli was again plated for induction of somatic embryos and subsequently for elongation of somatic into plantlets. Eventually, it took several months to obtain a homoplasmic transgenic plant from the bombarded pro-embryogenic cells. Thus, plastid transformation via embryogenesis is a slow process but it is quite reproducible and has been now demonstrated in several major crop species.

During these procedures, care was taken to maintain totipotency of transformed cells so that they do not lose the potential to form embryos. This was done by a combination of microscopic observations and constant evaluation of their embryogenic potential by inducing somatic embryos with suitable hormones. After obtaining homoplasmic cells that retained their potential to form somatic embryos, transgenic plants were produced from somatic embryos. Such transgenic plants were transferred to soil in pots for stabilization, establishment of the root system and further molecular characterization. Thus, the first successful chloroplast transformation via somatic embryogenesis was obtained by constructing suitable chloroplast vectors, optimization of DNA delivery and establishing conditions to achieve homoplasmy and regenerate stable transgenic plants. All scientific literature in the past has shown only one or two copies of foreign genes integrated into the nuclear genome or one or two transformed chloroplasts. For the first time, the applicant optimized a process in which transgenic plants regenerated via somatic embryogenesis contained cells that are homoplasmic (up to 10,000 copies of foreign genes per cell).

More specifically regarding the subject vector claims, there are several reasons that have impeded the extension of chloroplast transformation technology to several other plant species. Chloroplast transgenic lines are routinely obtained in tobacco via organogenesis. The chloroplast transformation vectors utilize homologous flanking regions for recombination and insertion of foreign genes. The vectors employed for chloroplast transformation of potato, tomato and *Lesquerella* contained the flanking sequences from tobacco or *Arabidopsis* (Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Skarjinskaia *et al.*, 2003). This may be one of the reasons for lower transformation

efficiency in these crops. Efficiency of tobacco plastid transformation using 100% homologous native flanking sequences has been quite high (Fernandez San Millan *et al.*, 2003; Dhingra and Daniell, 2004). However, when petunia flanking sequences were used for chloroplast transformation of tobacco, the transformation efficiency decreased drastically (DeGray *et al.*, 2001). The applicant achieved efficient transformation of carrot, cotton or other chloroplast genomes via somatic embryogenesis using species-specific chloroplast vector containing 100% homologous flanking sequences. Therefore, species-specific vectors have been used for demonstration of plastid transformation via somatic embryogenesis.

The use of non-green explants has often been cited as one of the major obstacles that has limited the chloroplast transformation to solanaceous crops (Bogorad 2000). Non-green tissues contain several kinds of plastids namely proplastids, leucoplasts, amyloplasts, etioplasts, chromoplasts, elaioplasts and gerontoplasts in which gene expression and gene regulation systems are quite different from green chloroplasts. In carrot plastid transformation, the expression cassette for the detoxification of the selection agent is functional in non-green cells due to the full length P*rrn* promoter used in the cassette that has binding sites for both the nuclear encoded and plastid encoded RNA polymerase (Daniell et al., 2002a; Devine and Daniell 2004). Previous studies have reported 100-fold less foreign gene (GFP) accumulation in amyloplasts of potato tubers compared to leaves was reported (Sidorov et al., 1999). In sharp contrast, in proplastids and in chromoplasts, 53.1% and 74.8% BADH activity respectively was observed when compared to leaf chloroplasts (100%). Such high level of transgene expression was achieved using appropriate heterologous regulatory sequences in the expression cassette. Both the selectable marker and the gene of interest (*aad*A and *badh* are transcribed by the plastid Prrn promoter; this 16S rRNA promoter drives the entire rRNA operon in the native chloroplast and contains binding sites for both the nuclear encoded and plastid encoded RNA polymerases (for a recent detailed review and discussion see Daniell et al., 2002a). Therefore, this promoter is capable of functioning in both proplastids and chloroplasts (green and non-green, in the light and dark). The *badh* gene is further regulated by the T7 gene 10 5'UTR capable of efficient translation in the dark, in proplastids present in non-green tissues. This is also the first report of stable transgene expression in proplastids. Transformation of carrot plastid genome is the very first

example of successful stable plastid transformation using non-green explants via somatic embryogenesis. Thus, the applicant now claims vectors for expression in non-green plastids.

The cotton chloroplast transformation vector accomplishes this by using genes coding for two different enzymes capable of detoxifying the same selection agent (or spectrum of selection agents), driven by regulatory signals that are functional in proplastids as well as in mature chloroplasts. Both *aph*A-*6* and *aph*A-*2* (*nptII*) genes code for enzymes that belong to the aminoglycoside phosphotransferase family but they originate from different prokaryotic organisms. Both enzymes have similar catalytic activity but the *aph*A-*6* gene product has an extended ability to detoxify kanamycin and provides a wider spectrum of aminoglycoside detoxification, including amikacin (Bateman and Purton, 2000; Huang *et al.,* 2002). In nuclear genetic engineering, majority of the crop species have been transformed using aminoglycoside detoxification (kanamycin for dicots and geneticin for monocots). Both transgenes (*aph*A*2* (*npt*II), *aph*A*6*) are transcribed by the full-length plastid Prrn promoter containing binding site for nuclear-encoded and plastid-encoded RNA polymerase and is expected to function both in proplastids and mature chloroplasts. The *aph*A-6 gene is further regulated by the T7 gene 10 5'UTR capable of efficient translation in the dark, in proplastids present in non-green tissues (*i.e.* grayish friable culture of cotton initially bombarded with cotton specific chloroplast vector). The *rps*16 3'UTR was used to stabilize *aph*A-6 gene transcripts (Stern and Gruissem, 1987). The T7 gene 10 5' UTR and rps16 3' UTR facilitated 74.8% transgene expression in non-green edible parts (carrots) containing chromoplasts (grown under the ground in the dark) and 48% in proplastids, compared to chloroplasts in leaves (100%, Kumar *et al.*, 2004). Therefore, the *aph*A6 gene is expressed in non-green and green plastids in the light or dark.

The nptII gene in the cotton plastid transformation vector is driven by the *psb*A 5' and *psb*A 3' UTRs, which have been repeatedly shown to be responsible for light regulated expression of transgenes integrated into the plastid genome (Dhingra *et al.*, 2004; Devine and Daniell, 2004; Daniell *et al.*, 2004; Fernandez San Millan *et al.*, 2003; Staub & Maliga, 1995). Thus, it is logical to expect a breakdown of kanamycin in both dark and light conditions Therefore, a combination of both *aph*A-*6* and *aph*A-*2* genes driven by regulatory signals in the light and in the dark, in both proplastids and chloroplasts, provides continuous protection for transformed plastids/chloroplasts from the selectable agent. These approaches helped in the optimization of transformation procedure

of cotton cell culture and achieve a high frequency of transformation. Such optimized conditions further helped to obtain transformation even with a single selectable marker gene (*aph*A-6).

The applicant respectfully traverses the §103(a) rejection in light of WO 99/10513 taken with McBride (USPN 5,925,806).

In light of the above discussion, it should be clear that McBride does not supplement the teachings of the art discussed above in such a way to have rendered obvious somatic embryogenesis as claimed. It is believed that McBride is sited as teaching the substitution and/or addition of a promoter and UTRs. AS explained above, there was much more to enabling somatic embryogenesis than this.

In light of all the foregoing, the withdrawal of all the prior art rejections is respectfully requested.

The Assistant Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 and 1.17 as required by this paper to Deposit Account 19-0065.

The applicant invites the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

The Commissioner is hereby authorized to charge any fees which may be required to Deposit Account No. 19-0065.

Respectfully submitted,

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JMS/mrc
Attachments: A Petition and Fee for Extension of Time Under 37 CFR §1.136(a)
Replacement Figures 2-3, 8-23, 25-31, and 38 (27 pages)